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Trop-2 inhibits prostate cancer cell adhesion to fibronectin through the $\beta 1$ integrin-RACK1 axis

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Abstract

Trop-2 is a transmembrane glycoprotein upregulated in several human carcinomas, including prostate cancer (PrCa). Trop-2 has been suggested to regulate cell-cell adhesion, given its high homology with the other member of the Trop family, Trop-1/EpCAM, and its ability to bind the tight junction proteins claudin-1 and claudin-7. However, a role for Trop-2 in cell adhesion to the extracellular matrix has never been postulated.

Here, we show for the first time that Trop-2's expression in PrCa cells correlates with their aggressiveness. Using either shRNA-mediated silencing of Trop-2 in cells that endogenously express it, or ectopic expression of Trop-2 in cells that do not express it, we show that Trop-2 inhibits PrCa cell adhesion to fibronectin (FN). In contrast, expression of another transmembrane receptor, $\alpha v\beta 5$ integrin, does not affect cell adhesion to this ligand. We find that Trop-2 does not modulate either protein or activation levels of the prominent FN receptors, $\beta 1$ integrins; however, it promotes $\beta 1$ association with the adaptor molecule RACK1, and causes significant redistribution of RACK1 to the cell membrane. As a result of Trop-2 expression, we also observe activation of Src and FAK, known to occur upon $\beta 1$ -RACK1 interaction. These enhanced Src and FAK activities are not mediated by changes in either the activity of IGF-IR, which is known to bind RACK1, or IGF-IR's ability to associate with $\beta 1$ integrins.

In summary, our data demonstrate that the transmembrane receptor Trop-2 is a regulator of PrCa cell adhesion to FN through the $\beta 1$ integrin-RACK1-Src-FAK signaling axis.

Keywords

Src; Focal adhesion kinase; Insulin-like Growth Factor I Receptor

Introduction

Integrins are transmembrane receptors for extracellular matrix (ECM) ligands, and are critical regulators of cell adhesion to ECM (DeRoock et al., 2001; Hynes, 2002; Alam et al.,

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2007). Several signaling molecules, including focal adhesion kinase (FAK), Src, extracellular signal-regulated kinase (ERK) and myosin light-chain kinase (MLCK) participate in the regulation of dynamic assembly and disassembly of focal adhesions, macromolecular platforms that contribute to stabilize cell attachment to the substrate (Kanchanawong et al., 2010). Aberrant integrin signaling, coupled with intensive proteolytic activity to degrade the surrounding ECM, is frequently detected in several diseases, including cancer metastasis. Moreover, regulation of the physical link between integrins and the actin cytoskeleton is critical for the spatio-temporal control of cell detachment from the substrate, cell migration and metastasis.

Trop-1/EpCAM and Trop-2 are members of a transmembrane glycoprotein family, which is upregulated in several human carcinomas as compared with their normal counterparts (Ohmachi et al., 2006; Zanna et al., 2007; Fong et al., 2008; Guerra et al., 2008; Trerotola et al., 2010). Trop-1 regulates epithelial cell-cell adhesion through homotypic protein-protein interactions (Balzar et al., 2001; Trebak et al., 2001) and modulates the activity of cadherins (Litvinov et al., 1997) through PI3K (Winter et al., 2007). Trop-2 is a Ca^{2+} signal transducer (Ripani et al., 1998), and has been suggested to resemble Trop-1 in cell-cell adhesion, since it binds the tight junction proteins claudin-1 and claudin-7 (Nakatsukasa et al., 2010). The use of Trop-2 for identification and isolation of stem cells in normal and neoplastic prostate has been reported (Goldstein et al., 2008; Goldstein et al., 2010), suggesting a role for this molecule in cancer initiation and progression. This is in line with previous literature correlating Trop-2 expression with poor prognosis in several human carcinomas (Ohmachi et al., 2006; Fong et al., 2008), and also upregulation during prostate cancer (PrCa) progression (Calvo et al., 2002).

Signaling pathways previously shown to affect integrin-mediated cell adhesion and cytoskeletal rearrangements involve a cross-talk between FAK, Src and the receptor for activated C kinase 1 (RACK1). While FAK and Src have intrinsic kinase activities (Lu et al., 1997; Marx et al., 2001; Yeatman, 2004; Mitra et al., 2005), RACK1 is an adaptor molecule known to bind several key signaling proteins, such as activated PKCs (Ron et al., 1994), FAK (Kiely et al., 2009; Serrels et al., 2010) and Src (Chang et al., 2001). RACK1 activity as regulator of central, stable focal adhesions has been recently reported (Cox et al., 2003; Serrels et al., 2010), suggesting a critical function of this molecule in cell-ECM interaction. Focal adhesions bring together cytoskeletal and signalling proteins during the processes of cell attachment and spreading on ECM, and phosphorylation of FAK is a rapid event associated with the formation of these adhesive structures (Mitra and Schlaepfer, 2006). Tyr397 is a major autophosphorylation site in FAK, and phosphorylated FAK promotes binding and activation of Src (Mitra and Schlaepfer, 2006). However, in FAK-null cells reconstituted with Y397F-FAK or A430V-Src constitutively inactive mutants, adhesion disassembly is significantly slower than in control cells (Webb et al., 2004), indicating that an intact FAK-Src signaling axis is critical for inducing disassembly and turnover of integrin-based adhesion sites, an essential step in the metastatic cascade (Cox et al., 2006; Natarajan et al., 2006). A complex between RACK1, FAK and the cAMP-degrading phosphodiesterase PDE4D5 has been recently reported to localize selectively in nascent focal contacts, rather than in mature adhesions that are formed in cells stably attached to ECM, and to promote cell polarity (Serrels et al., 2010). It has been shown that the N-terminal region of RACK1 contains the FAK binding site (Kiely et al., 2009); RACK1 has been also demonstrated to associate with the cytoplasmic domain of several integrins, among them $\beta 1$ (Liliental and Chang, 1998; Buensuceso et al., 2001; Besson et al., 2002). The integrin binding domain of RACK1 is localized in the C-terminal portion; in this region, the Tyr-246 residue has been identified as a Src binding site (Chang et al., 2001). Finally, RACK1 has been demonstrated to associate with the Insulin-like Growth Factor I (IGF-I) receptor (IGF-IR), suggesting a role in the IGF signaling network (Hermanto et al., 2002;

Kiely et al., 2002). IGF-I and IGF-IR, known to play a positive role in PrCa (Wu et al., 2006; Zoubeidi et al., 2010), stimulate many signaling pathways upon binding to each other. Recent reports have shown that IGF-I binding to IGF-IR, which promotes the interaction between $\beta 1$ integrins and RACK1 in adherent cells (Kiely et al., 2005), induces displacement of Src from RACK1 (Kiely et al., 2002); when Src is dissociated from RACK1, its kinase activity is increased (Cox et al., 2003).

Here, we describe for the first time that Trop-2 inhibits cell adhesion to fibronectin (FN) in PrCa cells, and we identify the $\beta 1$ integrin-RACK1-FAK-Src as major signaling axis involved in this function. This study demonstrates that the transmembrane glycoprotein Trop-2 is a critical mediator of cell-ECM interaction. We propose that the mechanisms underlying these events may involve the control of cytoskeletal dynamics and the turnover of focal adhesions.

Materials and Methods

Reagents and Antibodies

FN – purified from whole blood as described in (Engvall and Ruoslahti, 1977) – and Poly-L-Lysine (PLL) (Sigma-Aldrich) were diluted in sterile phosphate buffer saline (PBS) for coating. IGF-I was purchased from R&D Systems. Antibodies (Abs) used for flow cytometry (FACS) analysis were as follows: mouse monoclonal Ab (mAb) 162.46.2 (ATCC number: HB-187) against human Trop-2; mouse mAbs TS2/16 (ATCC number: HB-243) and K20 (M0889, Dako Cytomation), and rat mAb 9EG7 (550531, BD Biosciences), all against $\beta 1$ integrins. For immunofluorescence the mAb C20 (610177, BD Transduction Laboratories) against RACK1 was used. Abs used for immunoprecipitation (IP) were as follows: mAb against $\beta 1$ (clone K20), and rabbit polyclonal Ab (pAb) against IGF-IR β (C20; sc-713, Santa Cruz Biotechnology). Abs used for immunoblotting (IB) were as follows: mAbs against $\beta 1$ integrins (clone C18; 610468, BD Transduction Laboratories), head domain of talin (talin-H) (clone TA205; MAB1676, Chemicon), RACK1 (C20), and total Src (clone L4A1, 2110, Cell Signaling); rabbit pAbs against Tyr397 phospho-FAK (pFAK) (44-624G, Invitrogen), FAK (C-20; sc-558, Santa Cruz Biotechnology), Tyr416 phospho-Src (pSrc) (2101, Cell Signaling), and pY20 (sc-508, Santa Cruz Biotechnology); a goat pAb against human Trop-2 (R&D Biosystems). Negative control Abs were: purified non-immune mouse IgG (Pierce/Thermo Fisher Scientific) and purified normal goat IgG (Santa Cruz Biotechnology).

Cells and Culture Conditions

LNCaP (ATCC) and C4-2B (UroCor, Inc.) cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) (Gemini Bioproducts), 1% non-essential aminoacids, 1 mM sodium pyruvate, 10 mM Hepes pH 7.3. PC3-1 and PC3-2 are sublines of the original PC3 line from ATCC, and have been described in (Akech et al., 2010); these cells were cultured in RPMI 1640 supplemented with 10% FBS. DU145 (ATCC) cells were cultured in DMEM medium supplemented with 10% FBS.

Constructs and Cell Transfection

The vector for expression of Trop-2 in mammalian cells was pEGFP-N1 from Clontech (Palo Alto, CA), devoid of the coding sequence of EGFP (p Δ EGFP). The human TROP2 cDNA (Fornaro et al., 1995) was amplified by PCR with primers FT2FP (gcgattctcgagtcggtccgctgtcc – XhoI) and RT2FP (gcgccggtaccaagctcggttccttc – KpnI) and subcloned in the p Δ EGFP vector. Transfection of PC3-2 and LNCaP cells was performed with Lipofectamine 2000 reagent (Invitrogen) following manufacturer instructions. Stable transfectants were selected and maintained in complete RPMI supplemented with G418 (100

μg/ml). Enrichment for stable transfectants was performed by flow cytometry, using a fluorescence activated cell sorter (FACSVantage, Becton Dickinson). PC3-2/β5 cells were generated by transfection of the human β5 integrin cDNA subcloned in the pBABE retroviral vector, followed by selection with puromycin (0.5 μg/ml). To generate DU145 cells with stable knockdown of endogenous Trop-2, two human Trop-2-directed pLKO.1 lentiviral small hairpin RNA (shRNA) plasmids were obtained from OpenBiosystems (Cat. n. RHS3979-9623602 and RHS3979-9623605). To generate lentiviral particles, HEK293FT cells in 6-well plates were co-transfected with 1.85 μg of pLKO.1-Trop-2-shRNA vector, 1.85 μg of pLP1 (gag/pol fusion protein), 0.75 μg of pLP2 (Rev protein) and 0.4 μg of pLP/VSVG (VSV-G envelope glycoprotein) plasmids using Lipofectamine 2000. Lentivirus-containing supernatants were harvested 48 h after transfection, followed by filtration through a 0.45 μm pore diameter filter, and used to infect cultures of DU145 cells. Stably infected cells were cultured in the presence of 1 μg/ml puromycin for at least one week, and expression of Trop-2 was analyzed by IB to confirm downregulation. One of the two Trop-2 shRNA plasmids (Open Biosystems Cat. n. RHS3979-9623602) was used as control shRNA for further experiments since it did not downregulate Trop-2 expression.

Flow Cytometry

Human cell lines and transfectants were subjected to single staining with primary Abs, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti mouse (GAM) secondary Abs. Fluorescence intensity was determined by FACS analysis on a FACSCalibur or a FACS LSRII instrument interfaced with CellQuest Pro software (BD Biosciences).

Activation of β1 integrins in PC3-2 cell transfectants

The β1 integrin activation assay was performed as described (Green et al., 2009). Briefly, PC3-2/Trop-2, PC3-2/Mock and PC3-2/β5 cell transfectants cultured in complete medium were washed once with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, and cultured for 5 h in serum-free medium. Cells were then harvested using 1 mM EDTA in PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. After extensive washes with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, cells were incubated for 30 min in Hepes buffer [20 mM Hepes pH 7.4, 150 mM NaCl, 1% bovine serum albumin, (BSA)]. Hepes buffer devoid of divalent cations was compared with buffer supplemented with 1 mM CaCl_2 / 1mM MgCl_2 , or supplemented with 0.2mM MnCl_2 . Then, cells were incubated for 30 min on ice in the same buffers described above, in the presence of the Ab K20 to quantify total β1 integrin expression levels, or the Ab 9EG7 to quantify the levels of active β1. A mouse IgG was used as a negative control Ab.

Generation of Protein Lysates and IB

Protein lysates were prepared by scraping cells in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM CaCl_2 , 1mM MgCl_2 , 1% NP-40, 1 mM benzamidine, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μM calpain inhibitor, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{O}_7\text{P}_2$. After 15 min incubation on ice, lysates were centrifuged at 12,000 g for 10 min. Supernatants were collected and protein content was determined using the DC Protein Assay Kit (Bio-Rad). Samples were subjected to 10% SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes for IB.

Immunoprecipitation

IP experiments were performed as follows: cells were lysed in the lysis buffer described above and pre-clearing was performed by two consecutive incubations with protein G-Sepharose at 4°C for 45 min. Binding to the specific Abs (see above) was performed by incubation at 4°C for 3 h, followed by incubation with protein G-Sepharose for 1 h at 4°C.

After six washes with lysis buffer, immunocomplexes were separated by reducing SDS-PAGE.

Activation of IGF-IR in PC3-2 cell transfectants

PC3-2/Trop-2, PC3-2/Mock and PC3-2/ β 5 cell transfectants were subjected to stimulation with IGF-I as described (Goel et al., 2004). Briefly, cells were starved in serum-free RPMI for 24h, then IGF-I was added at 100 ng/ml for 3 min. Cells were lysed as described above, and IP was performed using an Ab against IGF-IR β . The immunocomplexes were then separated by SDS-PAGE and analyzed by IB, using an Ab against phosphorylated tyrosine residues (pY20) and against IGF-IR β .

Activation of FAK in PC3-2 cell transfectants

For FAK activation assay, PC3-2 cell transfectants were trypsinized, and trypsin was inactivated by soybean trypsin inhibitor. After three washes with serum-free RPMI/1% BSA, cells were maintained in suspension for 30 min at 37°C. Then, cells were seeded on plastic dishes coated with FN (10 μ g/ml) or PLL (1 mg/ml), and incubated for 15 min at 37°C. After two washes with PBS, cells were lysed and separated by reducing SDS-PAGE; then, IB analysis was performed using an Ab against pFAK (Tyr397) and total FAK.

Cell Adhesion Assay

Coating of plastic surfaces was performed using FN (10 μ g/ml) or PLL (1 mg/ml) overnight at 4°C. Cell adhesion assays were performed as described (Zheng et al., 1999). Briefly, cells were trypsinized, and trypsin was inactivated by soybean trypsin inhibitor (Roche). After three washes with serum-free RPMI/1% BSA, cells were counted and seeded on 96-well plates for 1 h at 37°C. Cells were later fixed with 3.7% paraformaldehyde (PFA) and stained with 0.1% crystal violet. Cell adhesion was quantified by absorbance at 562 nm using a microtiter plate reader (ICN Titertek Multiskan Bichromatic). Fisher's exact test was used for statistical analysis. Each experiment was performed in triplicate. The assays were repeated at least three times, and similar results were observed.

Immunofluorescence and Confocal Microscopy

Cells were seeded on FN-coated glass coverslips for 1 h at 37°C. Then, fixation with 3.7% PFA was performed for 15 min at room temperature, followed by quenching with 50 mM NH_4Cl . Cells were permeabilized by incubation with PBS / 0.2% Triton X-100 for 5 min, and then incubated for 30 min at RT with the blocking solution (PBS / 5% BSA). Staining was performed incubating samples with the primary mAb to RACK1 for 20 min at room temperature, followed by incubation with a secondary Ab (Alexa Fluor 488-Rabbit-anti-mouse) for 20 min at room temperature. After three washes, coverslips were mounted on glass slides using Pro-Long anti-fade reagent (Invitrogen), and slides were analyzed on an inverted confocal microscope (LSM510, Carl Zeiss) using Plan-Apochromat 63 \times (1.4 NA) or Plan-Neofluar 100 \times (1.3 NA) lenses.

Results

Trop-2 inhibits cell adhesion to fibronectin

We have previously shown that Trop-2, whose gene is designated *TACSTD2* (Fornaro et al., 1995), is upregulated in human PrCa (Trerotola et al., 2010; Trerotola et al., 2012); this upregulation is consistent with earlier reports demonstrating increased expression of Trop-2 in a murine model of PrCa progression (Calvo et al., 2002). As depicted in Figure 1, we extensively analyzed the expression levels of Trop-2 in five human PrCa cell lines. Three aggressive cell lines: PC3-1 [also designated PC3-H in (Akech et al., 2010)], C4-2B and

DU145; two less aggressive cell lines: PC3-2 [also designated PC3-L in (Akech et al., 2010)] and LNCaP. Trop-2 expression is found to be high in aggressive DU145 and PC3-1 cells, intermediate in C4-2B, and undetectable in PC3-2 and LNCaP. Thus, Trop-2's expression levels may reflect the aggressive phenotype of PrCa cells.

We silenced Trop-2 in DU145 cells using shRNA expressing lentiviruses; in parallel, we ectopically expressed Trop-2 in PC3-2 and LNCaP cells, as shown by IB (Fig. 2, right panels). Then, we seeded these cells on FN – a major component of the ECM – in order to evaluate Trop-2's effect on cell adhesion to extracellular substrates. As shown in Figure 2, we observe that shRNA-mediated silencing of Trop-2 enhances adhesion of DU145 cells to FN as compared with parental ($157.3 \pm 38.6\%$) and control shRNA cells ($77.4 \pm 19.3\%$) (Fig. 2A, left). Consistently, we observe that ectopic expression of Trop-2 significantly inhibits adhesion of PC3-2 cells to FN as compared with Mock ($55.8 \pm 4.8\%$) or $\beta 5$ ($68.0 \pm 4.0\%$) transfectants (Fig. 2B, left). Since $\alpha v \beta 5$ integrin is able to bind FN (Pasqualini et al., 1993), PC3-2/ $\beta 5$ transfectants were used as a control group in addition to PC3-2/Mock cells. We also observe that expression of Trop-2 in LNCaP cells inhibits adhesion to FN by $46.6 \pm 5.2\%$ as compared with Mock transfectants (Fig. 2C, left). In all cases, BSA was used as negative control and PLL as loading control.

This experimental evidence indicates that Trop-2 regulates PrCa cell adhesion to FN, suggesting that its activity may impinge on signaling networks regulated by FN receptors, namely $\beta 1$ integrins (Pytela et al., 1985).

Trop-2 does not affect $\beta 1$ integrin activation

We ruled out the possibility that $\beta 1$ integrin levels would be altered upon Trop-2 expression in PC3-2 transfectants. As shown in Figure 3A, we do not detect significant changes in $\beta 1$ protein levels on the cell surface of PC3-2/Trop-2 transfectants as compared with control cells. Therefore, we hypothesized that the effect of Trop-2 on cell adhesion to FN might be a consequence of Trop-2-induced modulation of $\beta 1$ integrin affinity for FN. A conformational switch from low-affinity or inactive form to high-affinity or active form occurs upon integrin binding to their ligands, but can alternatively be induced with activation stimuli such as Mn^{2+} ions (Hynes, 2002). This conformational switch exposes CLIBS (cation-and-ligand-influenced binding site) epitopes that can be recognized by specific antibodies, such as 9EG7 (Bazzoni et al., 1995). We performed a cation-mediated $\beta 1$ activation assay by flow cytometry, using PC3-2 transfectants incubated in the presence of either Ca^{2+}/Mg^{2+} ions (that do not activate $\beta 1$) or Mn^{2+} ions (that do activate $\beta 1$) (Bazzoni et al., 1995). As depicted in Figure 3B, we do not observe significant changes of the 9EG7 epitope's levels in Trop-2 transfectants versus negative controls, indicating that ion-mediated activation of $\beta 1$ is not affected by Trop-2. Thus, other mechanisms occur to induce the Trop-2-dependent anti-adhesive phenotype of PrCa cells.

Trop-2 stimulates the association of $\beta 1$ integrins with RACK1, and the activity of Src

RACK1 is an adaptor molecule that has been previously shown to regulate integrin-dependent cell adhesion to ECM ligands (Serrels et al., 2010), and to associate with $\beta 1$ integrins (Liliental and Chang, 1998; Besson et al., 2002). We show that upon ectopic expression of Trop-2 in PC3-2 cells, the association of RACK1 to $\beta 1$ integrins is strikingly enhanced (Fig. 4A), although RACK1's expression levels remain constant (Fig. 4B). We also performed IP of Trop-2, but we did not detect interaction with RACK1 (data not shown). We then analyzed the localization of RACK1 in PC3-2 transfectants by immunofluorescence (Fig. 4C). Our data show that RACK1 accumulates in membrane compartments in PC3-2/Trop-2 cells, whereas it appears to be diffuse in the cytoplasm of PC3-2/Mock transfectants (Fig. 4C, left). The percentage of cells in which RACK1 is

localized in membrane regions was as follows: 82.5 ± 0.9 per cell in PC3-2/Trop-2 (n=140 cells) as compared with 13.1 ± 2.3 per cell in PC3-2/Mock (n=195 cells) transfectants (Figure 4C, right). Thus, these data suggest that Trop-2 induces accumulation of RACK1 in membrane regions, where this adaptor molecule may form a tight complex with $\beta 1$ integrins. Recent reports have shown that IGF-I, required for RACK1 to bind $\beta 1$ integrins (Kiely et al., 2005), induces displacement of Src from RACK1 (Kiely et al., 2002) with increase in its kinase activity (Cox et al., 2003). Therefore, we tested Src activity by immunoblotting using an Ab specifically targeting the active form of this kinase, pSrc (Tyr416); as depicted in Figure 4D, we find that the levels of pSrc (Tyr416) are enhanced in PC3-2/Trop-2 transfectants as compared with PC3-2/Mock cells. This appears to be consistent with a role of Trop-2 as a promoter of $\beta 1$ -RACK1 interaction.

Trop-2 stimulates the phosphorylation of FAK

Adhesion signals and IGF-I promote association between IGF-IR, RACK1 and $\beta 1$ integrins (Kiely et al., 2005). Since IGF-IR plays a critical role in the regulation of RACK1's binding to several partners, we hypothesized that Trop-2 might regulate the activity of IGF-IR, or alternatively its interaction with $\beta 1$ integrins (Goel et al., 2004). As shown in Figure 5A, we observe that IGF-IR β expression levels are not affected by Trop-2. In addition, we find that Trop-2 does not alter the activity of IGF-IR. Indeed, when we incubate PC3-2 transfectants with IGF-I, rapid phosphorylation of IGF-IR β is detected, independently on Trop-2's expression (Fig. 5B). Consistently, the association between $\beta 1$ and IGF-IR was again unaffected when comparing PC3-2/Trop-2 with PC3-2/Mock and PC3-2/ $\beta 5$ cells (Fig. 5C).

Reduced cell adhesion to ECM substrates occurs through modulation of focal adhesion turnover, an event affected by FAK, a kinase known to associate with RACK1. Therefore, we investigated whether Trop-2 affects the activation of FAK in PC3-2 transfectants upon adhesion to FN. We observe that ectopic expression of Trop-2 induces phosphorylation of FAK (Tyr397) on FN, whereas reduced levels of phosphorylated FAK (Tyr397) are observed in PC3-2/Mock and PC3-2/ $\beta 5$ transfectants (Fig. 6A). Thus, Trop-2 promotes an anti-adhesive phenotype of PrCa cells modulating the $\beta 1$ integrin-RACK1-FAK-Src signaling axis.

Discussion

In this study, we demonstrate that Trop-2, a transmembrane protein that is frequently upregulated in human carcinomas, inhibits PrCa cell adhesion to FN. The mechanism underlying Trop-2's function in cell adhesion involves modulation of the $\beta 1$ integrin-RACK1-FAK-Src signaling axis.

This study describes a function for Trop-2 that has never been demonstrated before, in inhibiting cell adhesion to FN. This observation is relevant to the understanding of PrCa mechanisms since Trop-2 expression correlates with, and appears to promote, an aggressive phenotype of PrCa cells. Since it is conceivable that more aggressive cells need to be poorly adhesive and more motile than less aggressive cells, we conclude that Trop-2 expression is a requirement for cancer cells which need to detach from the ECM. Finally, this finding is also relevant to PrCa since $\beta 1$ integrins, receptors for FN, are known to be upregulated in this disease (Goel et al., 2008); thus, we speculate that Trop-2 expression is also required in distant sites, where FN is upregulated (Ruoslahti, 1999).

The signaling pathway, described here, by which Trop-2 affects PrCa cell adhesion to FN is also novel for the following reasons. First, it is independent of changes occurring in $\beta 1$ affinity, as shown by analysis of $\beta 1$ integrins from a "closed" conformation that does not allow binding to their ligands to an "open" conformation, resulting in increased affinity for

the ligands (Hynes, 2002). Second, we demonstrate that Trop-2's effects on PrCa cell adhesion to FN comprise relocalization of the adaptor molecule RACK1 from cytoplasm to the cell membrane, and, more importantly, enhanced association between $\beta 1$ integrins and RACK1. RACK1 has been reported to bind other integrin β subunits, including $\beta 5$ (Liliental and Chang, 1998; Buensuceso et al., 2001); however, ectopic expression of $\beta 5$ integrin in PC3-2 cells did not result in significant effects either on cell adhesion to FN or on the $\beta 1$ -RACK1 binding. This evidence indicates that Trop-2's effect is specific and cannot be obtained by expression of another transmembrane receptor. Third, Trop-2 does not alter the association between $\beta 1$ and IGF-IR, known to occur in PrCa cells (Goel et al., 2004), but its effect on $\beta 1$ -RACK1 appears to resemble either non-adhesive phenotypes (Kiely et al., 2005), or the ectopic expression of a defective Y1250/1251F IGF-IR mutant that does not bind RACK1, which make the $\beta 1$ -RACK1 association constitutive and insensitive to IGF-I stimulation (Kiely et al., 2005). We thus propose that cells may use Trop-2 to increase the association between $\beta 1$ integrins and RACK1 when they need to detach and invade the surrounding ECM. Finally, the novelty of our observation is that Trop-2 activates a RACK1-mediated signal from $\beta 1$ integrin-dependent adhesion to ECM ligands, which converges in the activation of the motility kinases FAK and Src (Chang et al., 1998; Mitra et al., 2005; Kiely et al., 2009; Serrels et al., 2010). This is consistent with a previous report showing that enhanced $\beta 1$ -RACK1 association contributes to activate FAK and Src (Kiely et al., 2005). Furthermore, additional studies have shown that the N-terminal region of RACK1 contains the FAK binding site (Kiely et al., 2009), whereas the C-terminal portion of RACK1 mediates interactions with $\beta 1$ integrins (Liliental and Chang, 1998; Buensuceso et al., 2001; Besson et al., 2002) as well as Src (Chang et al., 2001). Thus, while FAK is constitutively associated with RACK1 (Kiely et al., 2009), $\beta 1$ integrins and Src compete with each other for binding to the same region. As seen in the schematic model in Figure 6B, we propose that, as a consequence of Trop-2-mediated increase of the association between RACK1 and $\beta 1$, FAK is translocated in close proximity of the $\beta 1$ cytoplasmic tail. Then, FAK is autophosphorylated in response to $\beta 1$ signaling (Mitra and Schlaepfer, 2006), and Src is released in the cytoplasm and its activity is induced. Increased levels of pFAK (Tyr397) stimulate binding to active Src, that can further phosphorylate FAK in additional sites, such as Tyr925, to induce full activation and focal adhesion turnover. The less adhesive phenotype of PrCa cells observed upon expression of Trop-2 is consistent with earlier studies showing that a fully-functional FAK-Src pathway is necessary for disassembly and turnover of focal adhesions, and for a dynamic regulation of cell-ECM interaction (Webb et al., 2004), one of the earliest steps in the metastatic cascade. FAK has been described to control microtubule polarization within cells (Palazzo et al., 2004), and this phenotype has been linked to the cross-talk between FAK and Rho GTPases (Ren et al., 2000). Rac/Rho/Cdc42 GTPases control formation and disassembly of actin cytoskeletal structures (such as stress fibers, lamellipodia and filopodia) (Clark et al., 1998; Kuhn et al., 1998; Price et al., 1998; Hirsch et al., 2002), and support cell motility (Symons and Settleman, 2000). Rho-family GTPases are also known to promote cell migration (Wells et al., 2010) by activating p21-activated Ser/Thr kinases (PAK)s (Kumar et al., 2006; Acconcia et al., 2007), which cross-talk with Src (Siu et al., 2010). Given our evidence that Trop-2 impinges on networks regulated by FAK/Src, we envision a role for Trop-2 in PrCa cell migration through cross-talk with these major signaling hubs. Although the pathway described above appears to be predominant, other important signaling molecules like Pten, Akt (Goswami et al., 2005) and AR (Lamoureux et al., 2011) are critical for development and progression of PrCa. These molecules are therefore promising candidates for future investigations of additional molecular mechanisms underlying Trop-2-mediated events in advanced PrCa.

In summary, we describe a novel molecular mechanism of regulation of PrCa cell adhesion to FN, whereby the transmembrane glycoprotein Trop-2, previously suggested to have a role in cell-cell adhesion, modulates cell-ECM attachment by controlling the $\beta 1$ integrin-RACK1

complex and consequently FAK and Src. Further investigations are needed to delineate additional roles for Trop-2 in integrin-related functions, such as migration and invasion, and will allow to develop potential innovative drugs for target therapy of advanced PrCa.

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List of Abbreviations

ECM	extracellular matrix
FN	fibronectin
IP	immunoprecipitation
IB	immunoblotting
IF	immunofluorescence
FACS	flow cytometry
FAK	focal adhesion kinase
pFAK	phospho FAK
Src	c-Src
pSrc	phospho c-Src
ERK	extracellular signal-regulated kinase
IGF-I	Insulin-like Growth Factor I
IGF-IR	IGF-I Receptor
MLCK	myosin light-chain kinase
RACK1	receptor for activated C kinase 1
shRNA	small hairpin RNA
siRNA	small interfering RNA
PKC	protein kinase C
PLL	poly-L-lysine
PBS	phosphate buffer saline
FITC	fluorescein isothiocyanate
Ab	antibody
mAb	monoclonal Ab

pAb	polyclonal Ab
FBS	fetal bovine serum
GAM	goat anti mouse
PFA	paraformaldehyde
BSA	bovine serum albumin
SEM	standard error of the mean

List of Abbreviations used in Figures and Legends

Ab	Antibody
BSA	Bovine Serum Albumin
ctr shRNA	control shRNA
FACS	flow cytometry
FAK	focal adhesion kinase
FN	fibronectin
IB	immunoblotting
IF	immunofluorescence
IGF	insulin growth factor I
IGF-IRβ	IGF-I Receptor β
IgG	immunoglobulin G
IP	immunoprecipitation
mAb	monoclonal Ab
Neg. ctr. Ab.	negative control Ab
PBS	phosphate buffer saline
pFAK	phospho-FAK
PLL	Poly-L-Lysine
PrCa	Prostate Cancer
pSrc	phospho-Src
SEM	standard error of the mean
shRNA	small hairpin RNA
Susp.	suspension
Talin-H	talín-Head

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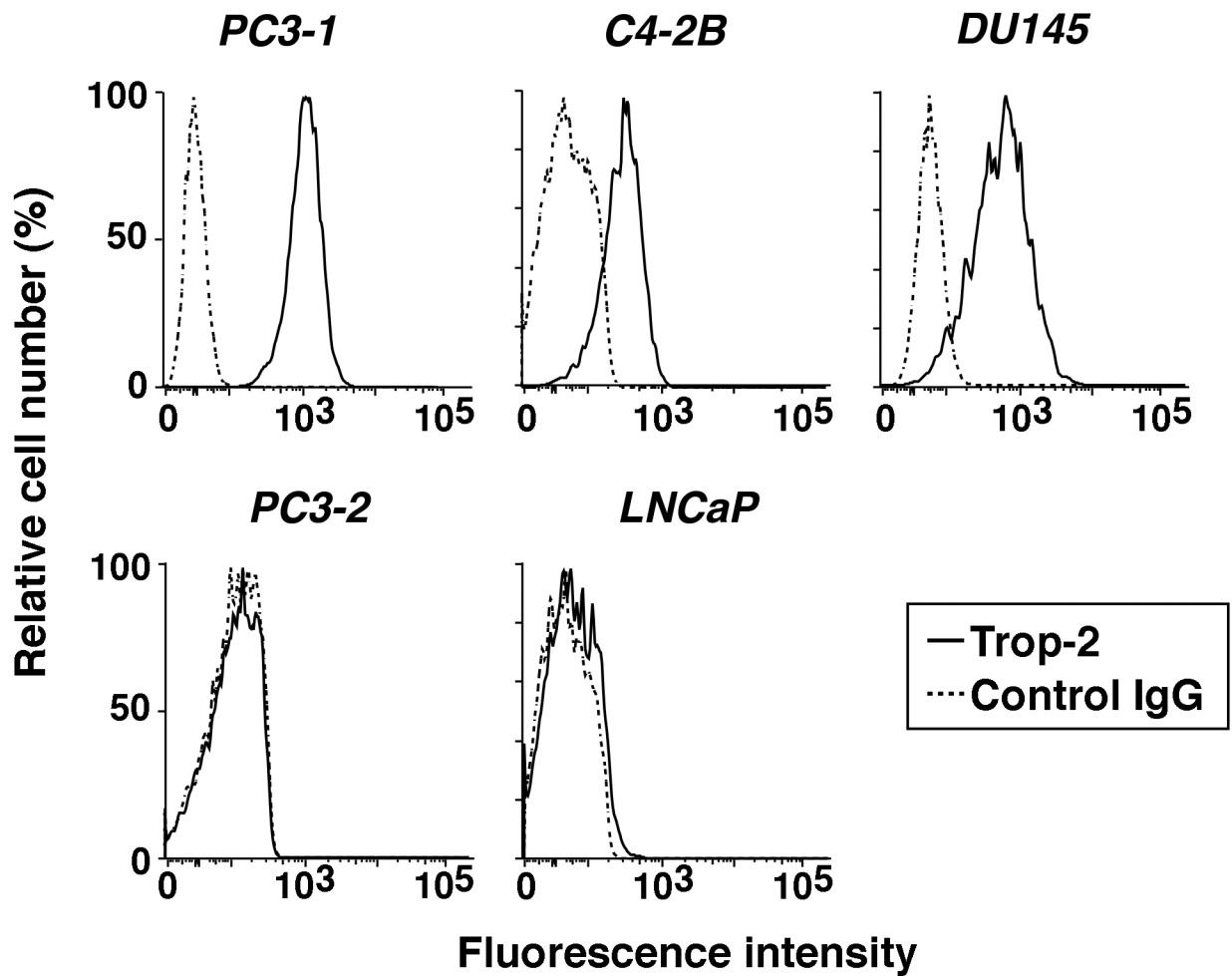


Fig. 1.

Trop-2 expression in PrCa cell lines. Surface expression profiles of Trop-2 were obtained by FACS analysis in five human PrCa cell lines: PC3-1, C4-2B, DU145, PC3-2 and LNCaP. Fluorescence intensity average values up to 10^3 and over 10^3 were chosen as thresholds to designate “intermediate” and “high” expression levels, respectively; “undetectable” expression was designated for profiles overlapping the ones obtained by staining with a negative control Ab. Profiles obtained by staining with a mAb to Trop-2, continuous lines. Profiles obtained by staining with a mouse IgG (negative control Ab), dotted lines.

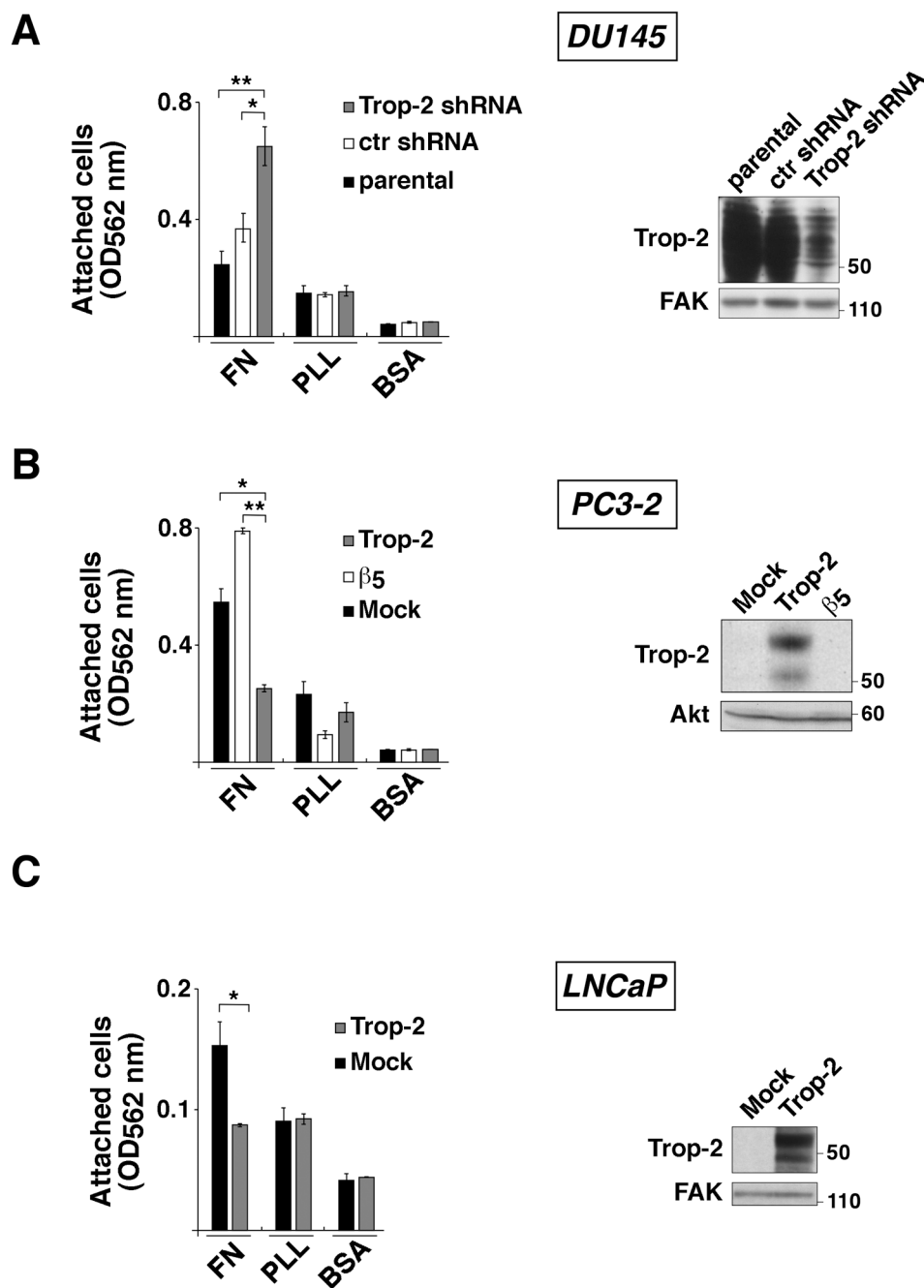
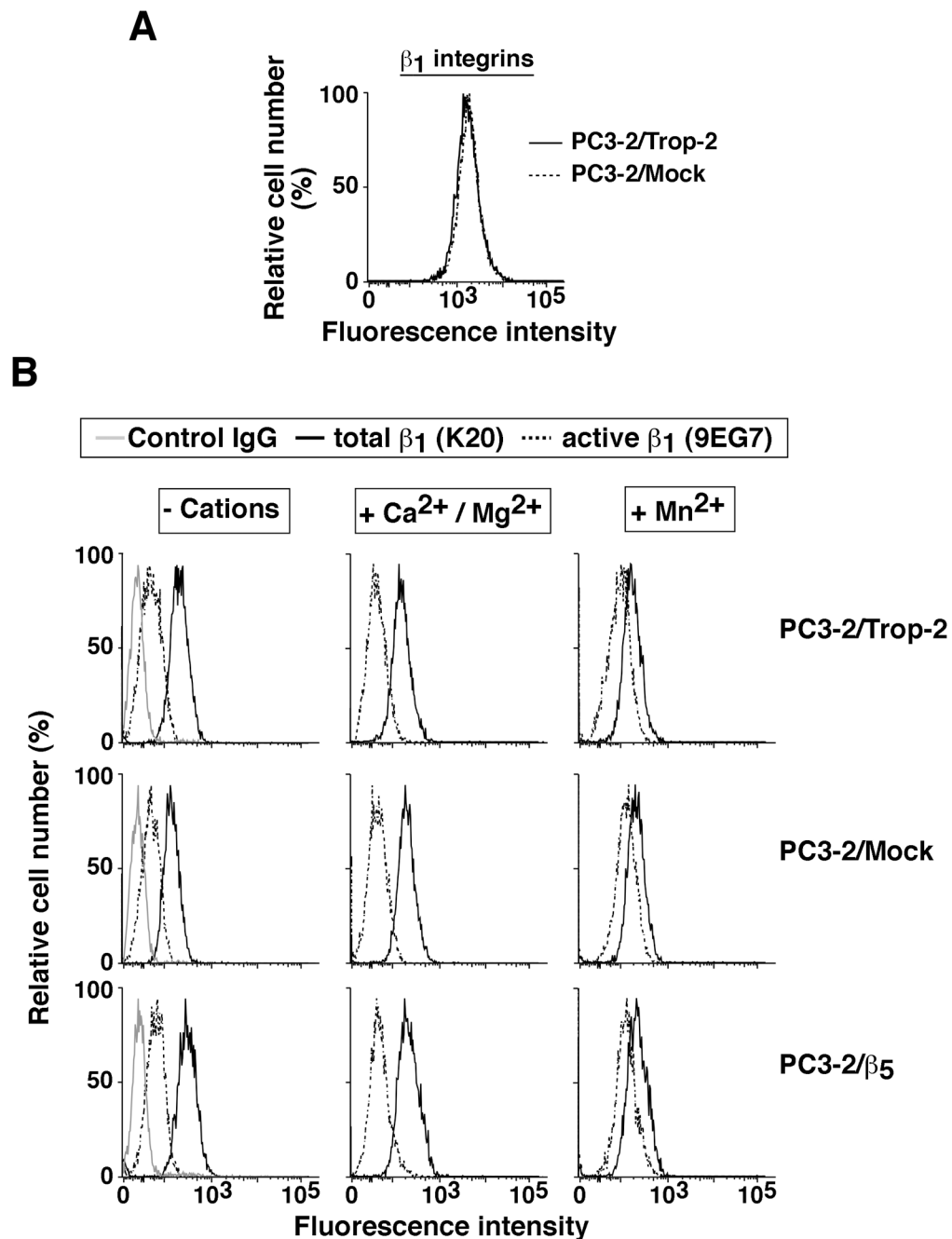


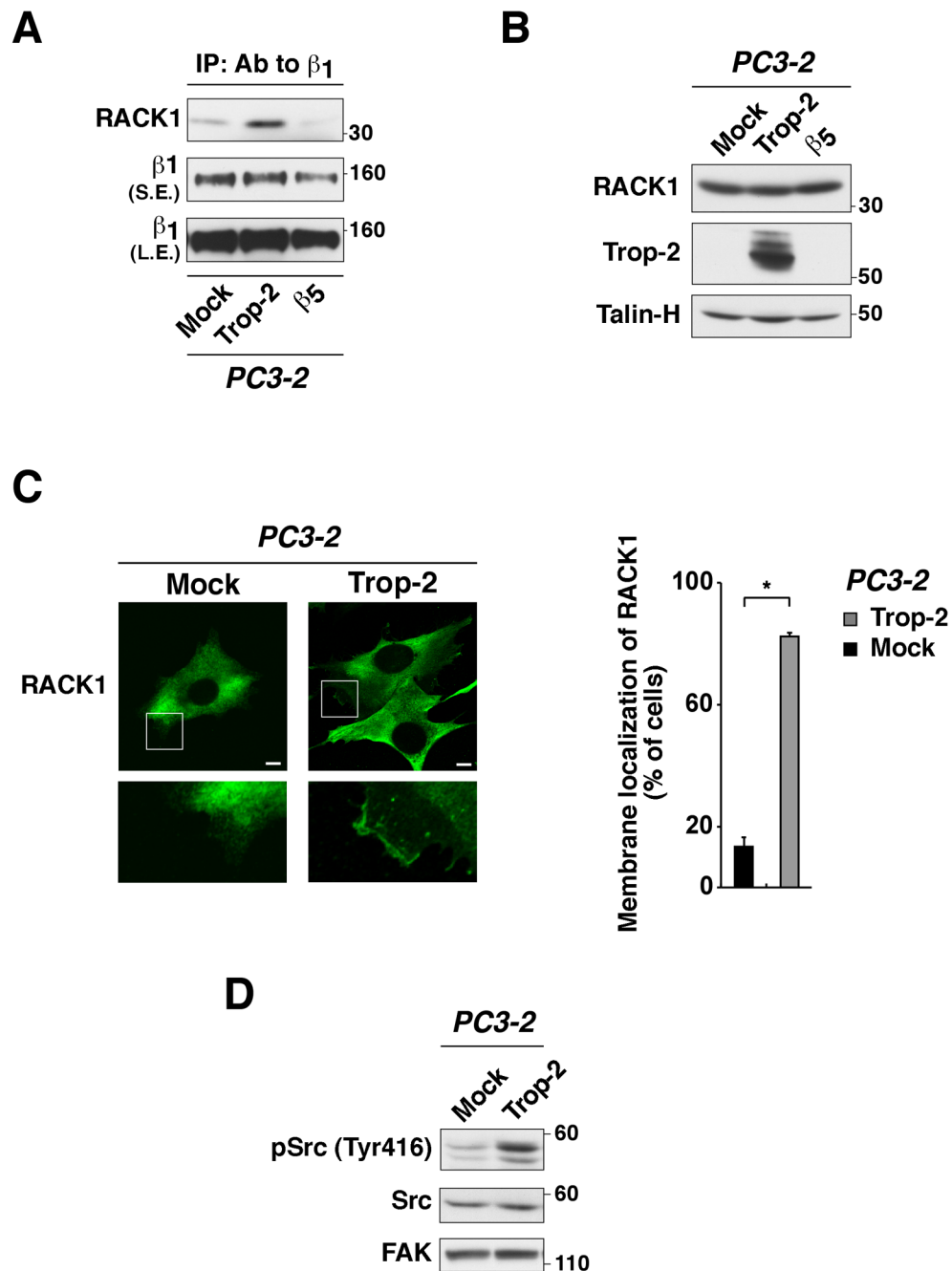
Fig. 2. Trop-2 inhibits cell adhesion to FN. (A) Adhesion assays were performed using DU145/Trop-2 shRNA cells seeded on FN, PLL and BSA, as described in Materials and Methods. Parental DU145 cells or DU145/ctr shRNA (infected with a non-silencing shRNA) were used as negative controls. Error bars, SEM. *, $P=0.021$. **, $P=0.004$. Silencing of Trop-2 was evaluated by IB (right panel). FAK, control of protein loading. (B) Adhesion assays were performed using PC3-2/Trop-2 cell transfectants seeded on FN, PLL and BSA. PC3-2/Mock and PC3-2/β5 transfectants were used as negative control groups. Error bars, SEM. *, $P=0.0095$; **, $P=0.0011$. Ectopic expression of Trop-2 obtained by transfection of PC3-2 cells was evaluated by IB (right panel). Akt, control of protein loading. (C) Adhesion assays

were performed using LNCaP/Trop-2 versus LNCaP/Mock transfectants. Error bars, SEM. *, $P=0.030$. Ectopic expression of Trop-2 obtained by transfection of LNCaP cells was evaluated by IB (right panel). FAK, control of protein loading.

**Fig. 3.**

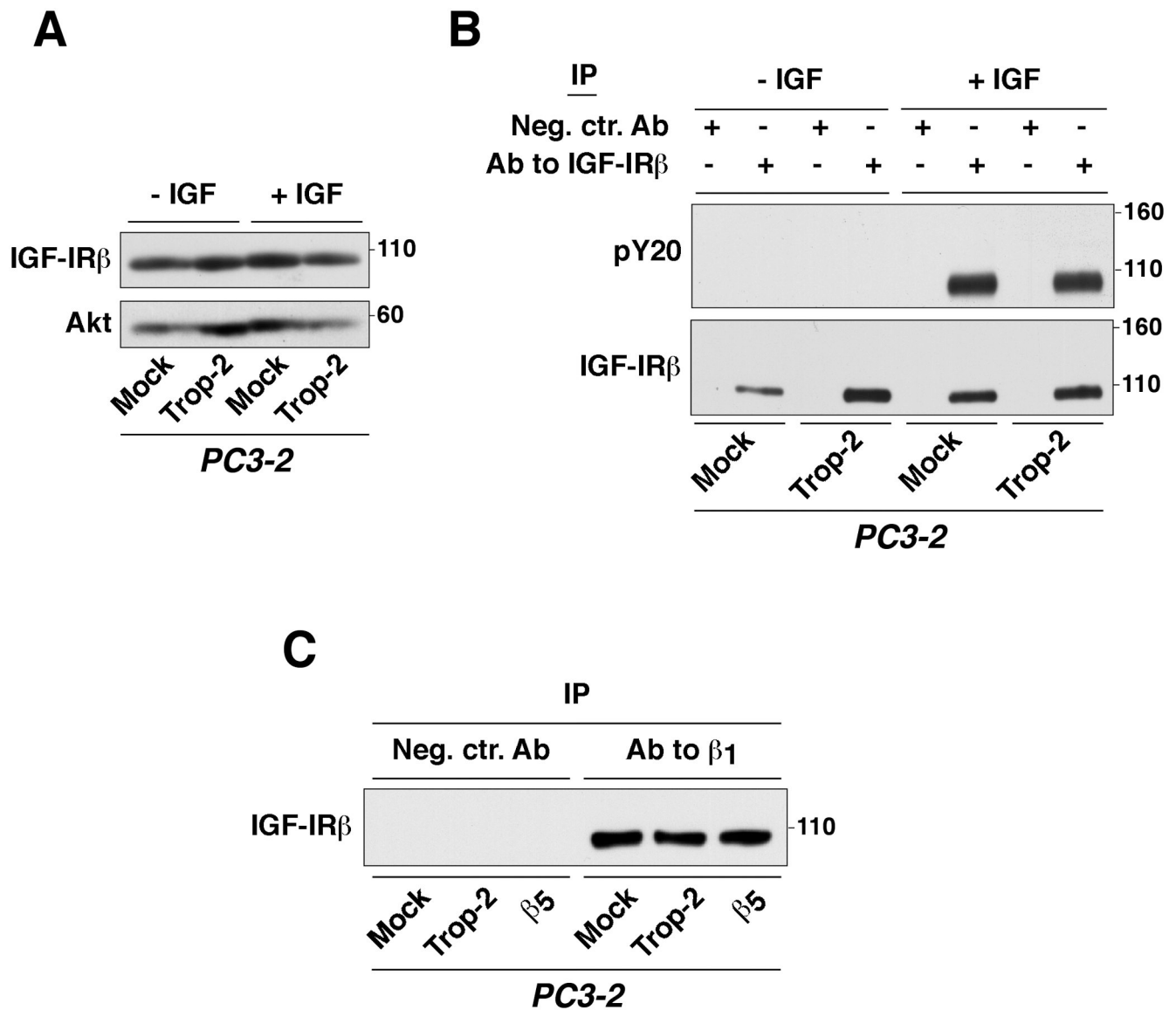
Ectopic expression of Trop-2 does not affect either β_1 integrin levels or activation. (A) β_1 integrin surface levels were analyzed by FACS. Continuous and dotted lines represent β_1 integrin surface expression profiles in PC3-2/Trop-2 and PC3-2/Mock cells, respectively. (B) Activation of β_1 integrins was evaluated by FACS analysis on PC3-2/Trop-2 (top panels) versus PC3-2/Mock (middle panels) and PC3-2/ β_5 (bottom panels) cells. Continuous black lines, total β_1 integrin expression profiles as measured by staining with K20 Ab. Dotted black lines, active β_1 integrin levels as measured by staining with 9EG7 Ab. Continuous gray lines, profiles obtained by staining with a negative control Ab. Left column, β_1 integrin expression profiles obtained by incubating cells in a buffer devoid of

divalent cations. Middle column, $\beta 1$ integrin expression profiles obtained by incubating cells in a buffer supplemented with CaCl_2 and MgCl_2 . Right column, $\beta 1$ integrin expression profiles obtained by incubating cells in a buffer supplemented with MnCl_2 .

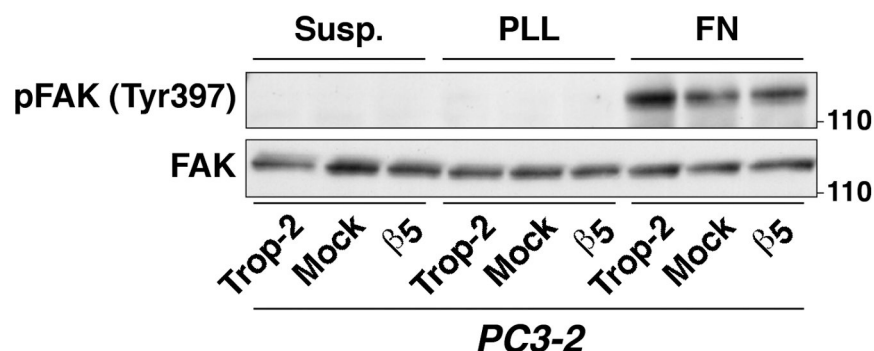
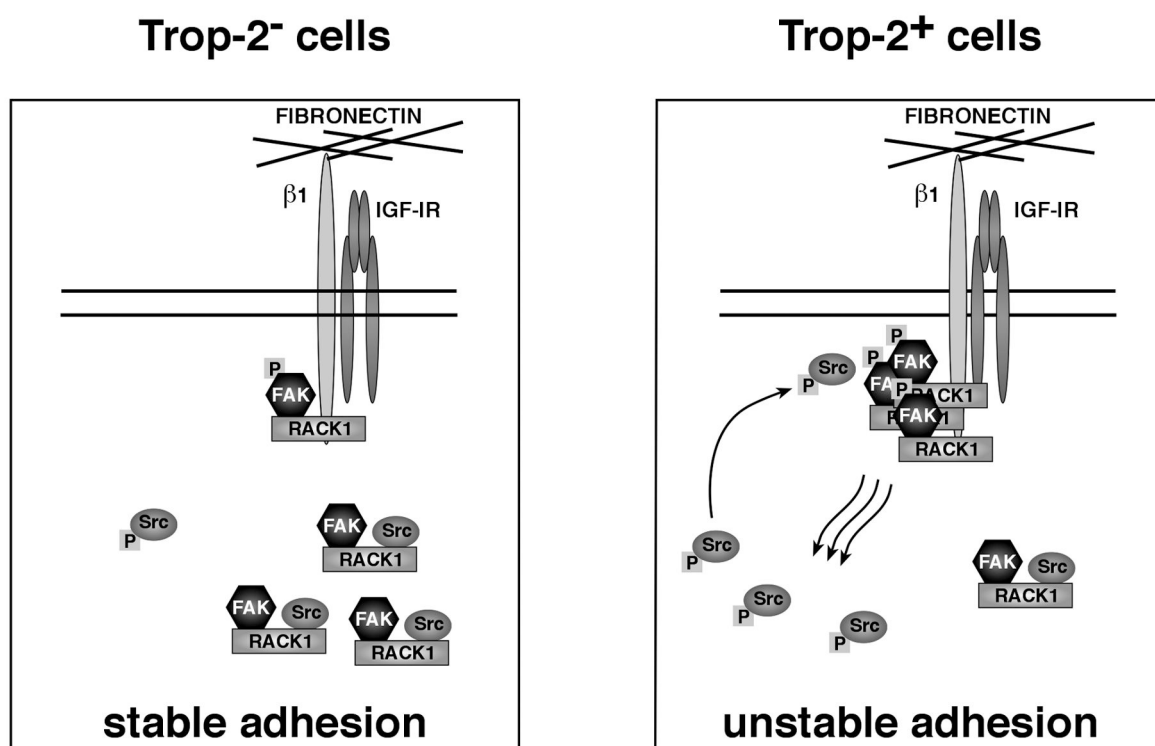
**Fig. 4.**

Trop-2 stimulates the association of β_1 integrins with RACK1, and activates Src. (A) β_1 integrins were immunoprecipitated using PC3-2 transfectants, and IB was performed on the immunoprecipitates using an Ab against RACK1. PC3-2/Trop-2 cells were compared with Mock and β_5 integrin transfectants in order to ensure specificity. IB using a mAb against β_1 was performed on the same immunoprecipitates as control of loading. (B) Expression levels of RACK1 were evaluated by IB in PC3-2/Trop-2 versus PC3-2/Mock and PC3-2/ β_5 transfectants. Talin-H, control of protein loading. (C, left) PC3-2/Mock and PC3-2/Trop-2 transfectants were seeded on FN and analyzed by IF staining with a mAb against RACK1. High magnification boxed areas (bottom) show membrane regions with specific localization

of RACK1. Bars, 10 μm . (C, right) The presence of RACK1 in membrane rims was evaluated. The bar graph indicates the percent fraction of cells showing membrane staining of RACK1 in PC3-2/Mock versus PC3-2/Trop-2 transfectants. Error bars, SEM. *, $P=0.0092$. (D) The activation of Src was analyzed by IB using an Ab against pSrc (Tyr416). PC3-2/Trop-2 transfectants were compared with PC3-2/Mock. Total Src and FAK, controls of protein loading.

**Fig. 5.**

Trop-2 does not affect either the activity of IGF-IR or its association with $\beta 1$ integrins. (A) Expression levels of IGF-IR β were assessed by IB using PC3-2 transfectants stimulated with IGF-I for 3 min. (B) IGF-IR β was immunoprecipitated, and its phosphorylation levels were assessed by IB using a pY20 Ab. IB using an Ab against IGF-IR β was performed to confirm that the receptor was pulled-down from both non-stimulated and IGF-I-stimulated PC3-2 transfectants. (C) The presence of the $\beta 1$ integrin/IGF-IR complex was investigated in PC3-2/Trop-2 cells compared with negative control groups by IP of $\beta 1$ and analysis of IGF-IR β by IB.

A**B****Fig. 6.**

Trop-2 activates FAK. (A) The activation of FAK was investigated using PC3-2/Trop-2 versus PC3-2/Mock and PC3-2/ $\beta 5$ cells seeded on FN for 10 min. Cells seeded on FN- or PLL-coated plastic dishes were washed with PBS, lysed and subjected to analysis by IB using an Ab against pFAK (Tyr397). Total FAK, control of protein loading. (B) Schematic drawing of Trop-2-mediated inhibition of PrCa cell adhesion to FN through the $\beta 1$ integrin-RACK1 signaling axis. (B, left) In cells that do not express Trop-2, RACK1 is mostly localized in the cytoplasm, where it binds FAK and Src, and prevents Src to be activated; the functional consequence is a decreased focal adhesion turnover, with stabilized cell adhesion to FN. (B, right) In Trop-2-expressing cells, increased binding of RACK1 to $\beta 1$ brings FAK

in close proximity to $\beta 1$, and this results in activation of FAK by autophosphorylation of its Tyr397 residue, release of Src from the complex, and Src activation by phosphorylation of the Tyr416 residue. Active Src and FAK interact with each other and stimulate downstream events that finally determine faster turnover of adhesive structures and destabilized cell attachment to FN.